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Production of D-galacturonic acid from pomelo peel using the crude enzyme from recombinant *Trichoderma reesei* expressing a heterologous exopolygalacturonase gene

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ABSTRACT

D-galacturonic acid is a starting material for synthesizing biochemicals used in cosmetic, pharmaceutical, and fuel industries. Utilization of one-step enzymatic hydrolysis method for D-galacturonic acid production can reduce the use of toxic chemical and avoid high temperature in pectin extraction process. However, lack of efficient enzymes facilitating degradation of pectin-rich biomass into D-galacturonic acid limited an implementation of this process. In this study, a heterologous *exoPG* gene encoding exopolygalacturonase (exoPG), a key pectinase enzyme originating from *Aspergillus aculeatus*, was expressed in a well-known industrial cellulase-producing strain, *Trichoderma reesei* RUT-C30. Application of this crude enzyme in the pectin-rich pomelo peel hydrolysis at 60 °C, pH 6.0 for 48 h resulted in up to 151.1 mg/g D-galacturonic acid, which was approximately 6.1-fold higher than that obtained by the enzyme prepared from the parental strain. Further economic assessment of D-galacturonic acid production by using crude enzyme from the recombinant *T. reesei* revealed that the investment could be returned within 0.5–0.7 year, suggesting profitability of the developed process. The crude enzyme from the engineered *T. reesei* strain developed in this study promises a clean and sustainable production of D-galacturonic acid from low-cost agricultural waste.

1. Introduction

The concept of a circular bioeconomy, where various biowaste types are transformed into value-added products, has gained increasing attention over the years (Stegmann et al., 2020). The shift from chemical process to a greener biocatalysis process appear to be an important way to eliminate waste and avoid the use of toxic substances. Biocatalysts like enzymes are biodegradable and the biocatalytic processes can be performed under mild condition and produce less waste than the conventional chemical processes (Sheldon, 2016). Therefore, the waste biorefinery model using biotechnological processes could be considered as a clean and sustainable way to reduce massive wastes, decrease environmental pollution and improve economic value of wastes (Zabaniotou and Kamaterou, 2019).

It is desired that abundant and low-cost substrates are used to enable economic production. Various agricultural wastes have been employed as cheap substrates for producing a variety of commodity chemicals (Teigiserova et al., 2019), plastic composites (Wang et al., 2021), and other high-value substances (Elsayed et al., 2020). Among the agricultural wastes, pomelo peel has received increasing interest in the recent years as sources of various bioactive compounds (Tocmo et al., 2020). Pomelo (*Citrus maxima* or *Citrus grandis*) is the largest citrus fruit with a

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thick peel commonly found in Southeast Asia and some East Asian countries, such as China and Japan (Burana-osot et al., 2010). In Thailand, the annual production of pomelo has been reported to be over 120,000 t (Thailand's Department of Agriculture, 2019). The pomelo fruit is either eaten fresh or processed into juice. However, a substantial amount of pomelo peel is disposed as waste, resulting in environmental problems (Wandee et al., 2019). Pomelo peel is a lignocellulosic material that mainly consists of cellulose (16.5%, based on dry matter), hemicellulose (6.86%), and pectin (35.42%) (Huang et al., 2014). The spongy white peel of pomelo, called albedo, has been reported to be rich in pectin (Methacanon et al., 2014). Several studies have demonstrated the potential use of pomelo peels for pectin production (Wandee et al., 2019) and bio-sorbents (Shang et al., 2018). Since pomelo peel waste contains a low lignin content, its biodegradation does not need any thermochemical pretreatment method to destruct the lignin structure, which is required by other lignocellulosic substrates (Martins et al., 2020). Therefore, pomelo peel can be used as an abundant and cheap biomass substrate to produce biochemicals derived from pectin.

D-galacturonic acid (GalA) is a major monomer of pectin, accounting for approximately 70% of all pectin components (Mohnen, 2008). GalA can be used as an acidifying agent in the food industry (Bélafi-Bakó, 2007) and can be further converted into other high value-added products, such as meso-galactaric acid (Vidgren et al., 2020), L-galactonic acid (Kuivanen et al., 2012), and butanediol (Tai et al., 2016), which can be used in the cosmetic, pharmaceutical, and fuel industries. The production of GalA from pectin-rich biomass comprises two steps: (i) pectin extraction with dilute acid at a high temperature (Vriesmann et al., 2011) and (ii) hydrolysis of pectin into GalA by hydrothermal treatment (Miyazawa and Funazukuri, 2004). However, thermal or chemical hydrolysis can produce considerable amounts of chemical wastes while consuming a substantial amount of energy (Leijdekkers et al., 2013). Therefore, one-step enzymatic hydrolysis has been considered as an alternative method in clean production of D-galacturonic acid from sugar beet pulp by using a crude enzyme from *Aspergillus niger* at 30 °C, pH 4.5; however, there is limited yield in this process (Schafer et al., 2020). This can be due to the complexity of the plant cell wall polysaccharides, which require several enzymes, such as pectinases, cellulases, and xylanases, for the complete hydrolysis of pectin and its associated polysaccharides (cellulose and hemicellulose) into sugar products. Therefore, it is still challenging to develop an efficient enzyme cocktail suitable for the direct extraction of GalA from pectin-rich biomass.

Zhang et al. (2016) reported that cellulase and pectinase are required to release GalA from plant materials. Cellulases play crucial roles in the hydrolysis of cellulose polymers into sugars by destroying the plant cell wall structure, whereas pectinases are essential for liberating GalA from pectin. Among pectinolytic enzymes, exopolygalacturonase (exoPG; EC 3.2.1.67 and EC 3.2.1.82) hydrolyzes the α-(1–4) glycosidic bond between the two GalA residues of a pectin polysaccharide, leading to the release of GalA and dimeric GalA, respectively (Parisot et al., 2003). *Trichoderma reesei* is among the well-established fungus that secretes high cellulase levels with high protein secretion efficiency (120 g/L of total protein at the industrial scale) (Gupta et al., 2016). The hyper-cellulase-producing strain, *T. reesei* RUT-C30, and its derivatives have been used for the industrial production of cellulase (Meng et al., 2018). However, pectinolytic enzymes are poorly produced by *T. reesei* (Seiboth et al., 2011). In this aspect, *T. reesei* enzyme preparation was often supplemented with exogenous pectinases from *Aspergillus* species (Giovannoni et al., 2020).

In order to promote circular bioeconomy, eco-friendly and sustainable production of GalA should be developed. As stated above, one-step enzymatic hydrolysis method for GalA production can be performed at a milder condition compared to the two-step process. However, direct hydrolysis of complex biomass substrate into single units of GalA required actions of several enzyme components. In the previous work, GalA production from pectin-rich substrate employed enzyme from native microorganisms, providing a GalA yield of 97.8 mg/g biomass (Schafer et al., 2020). On the other hand, development of recombinant enzyme-producing strain to enhance GalA production and economic assessment of this enzymatic process has not been explored. To fill this research gap, an *exoPG* gene (EC 3.2.1.67) from *Aspergillus aculeatus* BCC 17849, a high pectinase-producing fungal strain, was cloned and expressed in the *T. reesei* RUT-C30 strain to improve the production of GalA. The hydrolysis of pomelo peels by the enzymes from the recombinant *T. reesei* produced a substantially higher yield of GalA than that of enzymes from the *T. reesei* host strain. The hydrolysis conditions, including temperature, initial pH, substrate concentration, and enzyme loading, were optimized to maximize GalA yield. This is the first report on overexpression of exoPG gene in industrial *T. reesei* strain that can improved the GalA yield from agricultural wastes such as pomelo peel waste. The developed process allows a clean production of GalA using enzymatic reaction under a mild condition. Using cheap and renewable pomelo-peel waste as a raw material can reduce environmental deterioration associated with disposal of this waste. Economic assessment revealed profitability of the process allowing sustainable production of GalA from agricultural wastes.

2. Materials and methods

To develop efficient enzyme system for GalA production from agricultural waste, exoPG enzyme was overexpressed in industrial cellulase-producing fungus, *T. reesei*. Pomelo peel was used as an agricultural waste substrate for low-cost GalA production. Economic analysis was conducted to obtain economic indicators including total investment cost and payback period, which allowed determination of the newly developed process feasibility.

The overall experimental procedure is illustrated in Fig. 1. The first part was to construct recombinant *T. reesei* expressing heterologous ExoPG enzyme, which was used for enzyme production. Then, the second part focuses on evaluation of the crude enzyme produced by the recombinant *T. reesei* in hydrolysis of pomelo peels for GalA production. The next part presented bioprocess optimization for the production of GalA. Finally, economic analysis was conducted in two different enzyme loading scenarios.

2.1. Microbial strains

Escherichia coli DH5α was used for DNA manipulation and plasmid propagation. The *T. reesei* RUT-C30 strain was kindly donated by the Agricultural Research Service (ARS) Culture Collection (Peroria, IL, USA). *Agrobacterium tumefaciens* AGL-1 was used for transformation into *T. reesei* RUT-C30 mutants. *Pichia pastoris* BMGC 210 was provided by the Thailand Bioresource Research Center (https://www.tbrcnetwork.org).

2.2. Plasmid construction

In order to improve the enzyme-producing strain for hydrolysis of pectin-rich material, *T. reesei* RUT-C30 was used as the host strain due to its high level cellulase production. To construct the recombinant *T. reesei* strain, the pCZF plasmid backbone was used to express the *A. aculeatus exoPG* gene under the control of the pyruvate decarboxylase 1 (*PDC1*) promoter (Zhang et al., 2018). The mature *exoPG* sequence from *A. aculeatus* BCC 17849 was synthesized with an N-terminal cellobiohydrolase 1 (*CBH1*) secretion signal, with or without the C-terminal histidine (*His*) tag. The *exoPG* codon was optimized for expression in *T. reesei*. The PCR products of the *exoPG* and *exoPG-His* genes were ligated into the pCZF plasmid using Seamless Cloning Master Mix (Sangon Biotech, Shanghai, China). Then, pCZF-*exoPG* or pCZF-*exoPG-His* was transformed into *A. tumefaciens* AGL-1. The AGL-1 strain harboring the required plasmid was selected on Luria-Bertani (LB) agar containing 100 μg/mL kanamycin and 50 μg/mL rifampicin

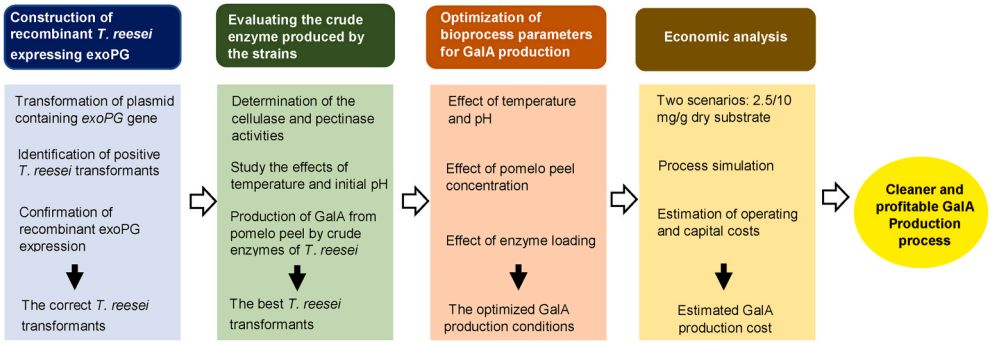

Fig. 1. Diagram of the experimental procedure of this study.

(Sigma-Aldrich, Darmstadt, Germany).

For the transformation of *T. reesei* using the *Agrobacterium*-mediated transformation method, the AGL-1 strains carrying pCZF-*exoPG* or pCZF-*exoPG-His* were co-cultured with *T. reesei* RUT-C30 conidia according to a previously described method (Zhang et al., 2018). The positive transformants were verified by polymerase chain reaction (PCR) using genomic DNA as the template with the following primers: forward, vCZF-F (5′-GTTTGTCCGAGCTGTTGATGGTTG-3′); reverse, vCZF-R (5′-CGACACCAACGATCTTATATCCAG-3′). The *T. reesei* transformants carrying pCZF-*exoPG* or pCZF-*exoPG-His* were designated ExoPG and ExoPG-His, respectively.

2.3. Enzyme production from recombinant T. reesei

To evaluate the recombinant strains, each *T. reesei* transformant was subjected to enzyme production under submerged condition. *T. reesei* strains were sub-cultured on malt extract agar (20 g/L each of malt extract and agar; Sangon Biotech) and incubated at 28 °C for 7 d to induce spore formation. The spores were resuspended in sterilized water, and 2×10^7 spores were inoculated into 1 L of Mandels medium (MM) (Mandels and Weber, 1969) containing KH_2PO_4, 2 g; $CaCl_2.2H_2O$, 0.4 g; urea, 0.3 g; $MgSO_4.7H_2O$, 0.3 g; $(NH_4)_2SO_4$, 1.4 g; $FeSO_4.7H_2O$, 0.005 g; $MnSO_4.H_2O$, 0.0017 g; $ZnSO_4.7H_2O$, 0.0014 g; $CoCl_2.2H_2O$, 0.002 g; proteose peptone, 1 g; Tween 80 0.2 g; lactose, 10 g. The precultures were grown at 28 °C with shaking at 200 rpm for 2 d. For enzyme production, the precultures were inoculated (8% v/v) with 50 mL of modified MM medium containing 1% (w/v) wheat bran in a 250-mL Erlenmeyer flask. The cultures were incubated at 28 °C with shaking at 200 rpm for 7 d. The crude enzyme was then separated from the solid materials and mycelium by filtration through nylon cloth, followed by centrifugation at 8,200×*g* at 4 °C for 10 min. The crude enzyme protein concentration was determined using Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA, USA) and bovine serum albumin (BSA) as a standard protein. All cultivations were performed in triplicate.

2.4. Production of exopolygalacturonase from P. pastoris

To compare the hydrolytic performance of the recombinant *T. reesei* enzyme preparation with the activity of exoPG, *P. pastoris* BMGC 210 harboring the mature *exoPG* gene from *A. aculeatus* BCC 17849 was used. The yeast strain was initially grown on yeast peptone dextrose (YPD) agar [2% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) dextrose, and 1.5% agar] at 30 °C for 24 h. A single colony was subsequently cultured in 5 mL YPD broth as the inoculum at 30 °C with shaking at 200 rpm for 24 h. For enzyme production, 0.1% inoculum was cultured in 25 mL of buffered complex glycerol (BMGY) medium [2% (w/v) peptone, 1% (w/v) yeast extract, 1% glycerol, 100 mM potassium phosphate (pH 6.0), 4×10^5% (w/v) biotin, and 1.34% yeast nitrogen base (YNB)] at 30 °C with shaking at 200 rpm for 18 h (OD_{600} = 5 to 6). The cells were harvested by centrifugation at 5,000×*g* at room temperature for 5 min. The cells were then continuously cultured in 5 mL buffered complex methanol (BMMY) medium [(2% (w/v) peptone, 1% (w/v) yeast extract, 100 mM potassium phosphate, (pH 6.0), 4×10^5% (w/v) biotin, 1.34% YNB, and 3% (v/v) methanol] at 30 °C with shaking at 200 rpm for 72 h. The enzyme-containing supernatant was separated from the cell by centrifugation at 5,000×*g* at 4 °C for 5 min.

2.5. Enzyme activity assays

The crude enzyme produced by each microbial strain was evaluated for polygalacturonase, cellulase [filter paper activity (FPase), carboxymethyl cellulase (CMCase), β-glucosidase (BGL)], and xylanase activities. FPase was determined using the method previously described by Ghose (1987). Briefly, the enzymatic reactions were carried out at 50 °C under mildly acidic conditions [1 × 6 cm cellulose (Whatman No.1 filter paper), 50 mM sodium citrate buffer, pH 4.8]. The CMCase, xylanase, and polygalacturonase activities were evaluated in a reaction containing 50 mM sodium acetate buffer (pH 5.0) using 1% (w/v) carboxymethyl cellulose, 1% (w/v) xylan from beech wood, and 0.5% (w/v) polygalacturonic acid (Sigma-Aldrich) as the substrates. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol/mL of glucose, xylose, or D-galacturonic acid per min under the assay conditions. For enzyme characterization, 50 mM glycine HCl buffer and 50 mM sodium phosphate buffer were used to determine the enzyme activity at pH 3.0–4.0 and 6.0–8.0, respectively. The 1-mL enzymatic reaction was conducted at 50 °C for 10 min. The liberated reducing sugars were quantitated using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The BGL activity was determined in a reaction containing 40 mM p-nitrophenyl-β-D-glucopyranoside (pNPG; Sigma-Aldrich) and 10 mM sodium acetate buffer (pH 5.0). The reaction was then incubated at 50 °C for 10 min. The enzymatic reaction was terminated using 100 mM sodium carbonate (Na_2CO_3). The reaction mixture (200 μL) was transferred to a 96-well plate, and the p-nitrophenolate concentration was determined at 405 nm (Suwannarangsee et al., 2014).

2.6. Production of D-galacturonic acid from pomelo peel waste

Production of GalA by crude enzyme from recombinant *T. reesei* and their original strain was conducted by using pomelo peel as a substrate. Pomelo white peel was kindly provided by Dr. Chaiwut Gamonpilas (National Center for Metal and Material Technology, Pathumthani, Thailand). The dried pomelo peel was ground and sieved into 1 mm particles using a SM2000 cutting mill (Retsch, Haan, Germany) (Methacanon et al., 2014). Enzymatic hydrolysis was carried out as previously described (Arnthong et al., 2020) with some modifications. The Eppendorf tubes contained 5% (w/v) pomelo peel, 50 mM sodium acetate buffer (pH 5.0), 1 mM sodium azide, and 2.5 mg protein/g dry substrate with a total volume of 1 mL. The hydrolysis mixture was incubated at 50 °C with shaking at 200 rpm for 48 h. All reactions were

carried out in quadruplicate. The total reducing sugars were determined using the DNS method (Miller, 1959). The amount of each reducing sugar was analyzed using a high-performance liquid chromatograph (HPLC) (Waters, Milford, MA, USA), equipped with a reflective index detector and an Aminex HPX-87H column (Bio-Rad) with a column temperature of 65 °C and a flow rate of 0.5 mL/min of 5 mM H_2SO_4.

For optimization of hydrolysis conditions, the effect of temperature was evaluated at 40, 50, and 60 °C in a reaction containing 5% (w/v) pomelo peel, 50 mM sodium acetate buffer (pH 5.0), 1 mM sodium azide, and 2.5 mg protein/g dry substrate with shaking at 200 rpm for 48 h. For the effect of pH, enzymatic hydrolysis was conducted at 50 °C under the same conditions as described above, except that 50 mM sodium acetate buffer (pH 4.0–6.0) or 50 mM sodium phosphate buffer (pH 6.5–7.0) was used. Thereafter, the hydrolysis reaction was set up at the optimum temperature (60 °C) and initial pH (pH 6.0), and the effects of solid loading (5.0, 10.0, and 15.0% (w/v)) and enzyme loading (2.5, 5.0, 7.5, and 10.0 mg protein/g dry substrate) on pomelo peel hydrolysis were investigated. The experiments were performed in quadruplicate. The GalA yields obtained with 5% (w/v) of substrate loading and enzyme loading of 2.5 and 10.0 mg protein/g dry substrate were used for economic assessment.

2.7. Economic analysis of D-galacturonic production

The economic analysis was performed to ensure technological feasibility of this newly developed process. Aspen Plus simulation software (version 11, Aspen Tech, USA) was used to evaluate the economic feasibility of the GalA production process. The physical and thermodynamic properties of pomelo peel were introduced to the software based on the experimental results. The pH adjustment during enzymatic hydrolysis of pomelo peel was assumed using either sulfuric acid or sodium hydroxide. Following the results of simulation, mass balance, quantities, and calculations of all the equipment were determined. Aspen PEA was applied for the estimation of equipment costs. To simulate the production of GalA, the feed of raw material was 350 kg/d of pomelo peel loading. Two different scenarios of enzyme loading to the enzymatic hydrolysis process were assessed. The scenario I and II refer to the hydrolysis reaction by using 2.5 and 10 mg protein/g dry substrate of enzyme loading, respectively. In this study, the desired internal rate of return was settled as 20%. Total capital investment cost, annual operation costs, and cash flow were calculated by Aspen PEA. The obtained results were used to determine the GalA production cost.

3. Results and discussion

3.1. Cloning and expression of exopolygalacturonase in T. reesei

To construct the engineered *T. reesei* overexpressing exopolygalacturonase as an enzyme producing strain for hydrolysis of pomelo peel, the plasmid pCZF-*exoPG* or pCZF-*exoPG-His* was introduced into the *T. reesei* RUT-C30 strain. The *T. reesei* transformants harboring pCZF-*exoPG* or pCZF-*exoPG-His* (Fig. 2A) were selected on potato dextrose agar (PDA) plates containing 100 μg/mL hygromycin B. Two and four positive clones of ExoPG and ExoPG-His transformants, respectively, were confirmed for the gene harboring PCR. A pair of primers, vCZF-F and vCZF-R, was used to amplify the DNA fragment to the expected size of approximately 1,896 bp, including the *exoPG* gene (1,296 bp) and partial plasmid backbone (600 bp). *T. reesei* RUT-C30 served as the negative control strain, in which the expected PCR product was absent. According to the results, one positive clone of *T. reesei* transformants harboring pCZF-*exoPG* was obtained and designated ExoPG 2. Three clones of *T. reesei* transformants containing pCZF-*exoPG-His* were acquired and designated ExoPG-His 1, ExoPG-His 2, and ExoPG-His 3 (Fig. 2B).

The expression of recombinant exoPG-His in *T. reesei* RUT-C30 was confirmed by the purification of crude enzymes from the ExoPG-His 1, ExoPG-His 2, and ExoPG-His 3 transformants using a Ni^{2+}-affinity column. The purified protein bands specific to Ni^{2+}, with a molecular weight of approximately 70 kDa, were present in ExoPG-His 1, ExoPG-His 2, and ExoPG-His 3 samples (Fig. S1). Western blot analysis was performed using the antibody against the His-tag. The positive band against the antibody corresponded to 70 kDa protein of the purified exoPG-His proteins, thereby confirming that these protein bands were exoPG-His proteins. These results indicated that heterologous exoPG-His was successfully expressed in *T. reesei* RUT-C30. In contrast, the ExoPG 2 transformant protein expression could not be detected by Western blotting due to the absence of the His-tag. The presence of recombinant exoPG protein in the crude enzyme was further investigated using an enzyme assay.

3.2. Profile of enzyme activities produced by T. reesei transformants

The enzyme production by recombinant *T. reesei* strains was determined to investigate the expression of heterologous exoPG as well as the background cellulase activity. After submerged cultivation, the supernatant was separated from the mycelium and used as a crude enzyme. Table 1 shows the protein concentrations and enzyme activities of the

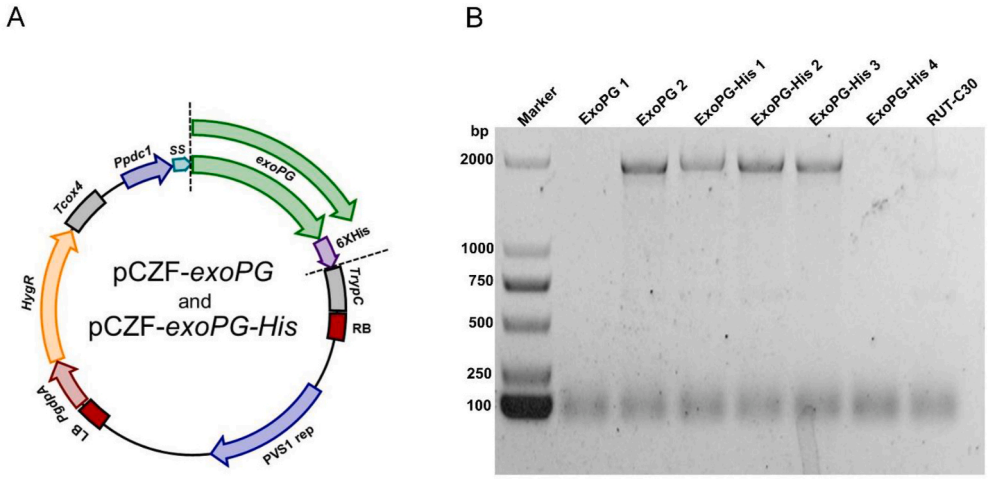

Fig. 2. Confirmation of pCZF-*exoPG* or pCZF-*exoPG-His* transformation into *T. reesei* RUT-C30 by colony polymerase chain reaction (PCR). (A) Plasmid construction of pCZF-*exoPG* and pCZF-*exoPG-His* (B) Confirmation of *exoPG* and *exoPG-His* gene harboring by PCR.

Table 1
Protein concentration and enzyme activities of the crude enzyme preparation from *Trichoderma reesei* strains and the recombinant exoPG enzyme produced by *Pichia pastoris* BMGC 210 (*P*exoPG). Data represent mean ± standard deviation (SD).

Strain	Protein (g/L)	PG[a] (U/mL)	FPase[a] (U/mL)	CMCase[a] (U/mL)	BGL[a] (U/mL)	Xylanase (U/mL)
RUT-C30	0.41 ± 0.03	15.17 ± 1.09	1.13 ± 0.05	45.10 ± 1.33	0.01 ± 0.00	11.78 ± 0.09
ExoPG2	0.51 ± 0.02	33.29 ± 3.56	1.31 ± 0.10	51.55 ± 0.27	0.02 ± 0.00	15.16 ± 1.21
ExoPG-His 1	0.55 ± 0.02	48.30 ± 0.35	1.46 ± 0.10	48.59 ± 3.23	0.02 ± 0.00	12.94 ± 1.59
ExoPG-His 2	0.55 ± 0.02	64.71 ± 2.55	1.21 ± 0.10	34.29 ± 0.86	0.06 ± 0.00	15.61 ± 1.02
ExoPG-His 3	0.58 ± 0.01	53.16 ± 1.26	1.57 ± 0.04	37.54 ± 5.00	0.02 ± 0.00	9.30 ± 0.31
*P*exoPG	0.24 ± 0.01	1,331.2 ± 43.0	N/D[b]	N/D[b]	N/D[b]	N/D[b]

[a] PG, polygalacturonase; CMCase, carboxymethyl cellulase; BGL, β-glucosidase; FPase, filter paper activity (total cellulase activity).
[b] N/D: enzyme activity was not detected.

crude enzymes from *T. reesei* transformants and their host strains. It was found that the protein concentration of all *T. reesei* transformant enzymes (0.51–0.58 g/L) was slightly higher than that of the RUT-C30 strain with a protein concentration of 0.41 g/L. The enzyme assay revealed that the *T. reesei* transformants exhibited at least 2-fold higher polygalacturonase activity (33.3–64.7 U/mL) than the host strain (15.2 U/mL). The highest polygalacturonase activity of 64.7 U/mL was achieved by the *T. reesei* ExoPG-His 2 strain, 4.3-fold higher than its original strain activity. In contrast, the enzymatic activities of CMCase, FPase, β-glucosidase, and xylanase were comparable between the host strain and the transformants. Among the cellulases, CMCase showed the highest enzymatic activity, whereas β-glucosidase showed the lowest (almost undetected). The lack of β-glucosidase activity in *T. reesei* has also been reported previously (Li et al., 2016). The remarkable improvement in polygalacturonase activity of the transformant crude enzymes implied that the exoPG and exoPG-His enzymes were efficiently expressed as secreted proteins by *T. reesei* RUT-C30. The presence of the His-tag at the exoPG C-terminus does not interfere with polygalacturonase activity and provides an advantage for the purification and detection of proteins. Moreover, the heterologous expression of exoPG in *T. reesei* RUT-C30 did not significantly affect the expression of the homologous cellulolytic enzymes.

3.3. Effects of temperature and initial pH on the activity of recombinant exoPG

In order to determine the optimum temperature and initial pH for the recombinant exoPG activity, the polygalacturonase activity of the enzymes secreted by the *T. reesei* ExoPG-His 2 strain was evaluated at different temperature and pH conditions. It was found that the polygalacturonase activity of ExoPG-His 2 crude enzyme increased with increasing temperature until it reached maximum activity at 60 °C—beyond this point, activity decreased (Fig. S2). In comparison, the optimum temperature for the *A. aculeatus* recombinant exoPG-His was slightly higher than that for the polygalacturonase enzymes from the other strains, with optimum temperatures ranging from 30 to 50 °C (Jayani et al., 2005). Under different pH conditions, polygalacturonase activity was observed under acidic conditions with an optimum pH of 4.0. However, increasing the pH to neutral and alkaline conditions (pH 6.0–8.0) resulted in the polygalacturonase becoming inactive (Fig. S2). According to other reports, acidic conditions in the pH range of 3.0–4.0 tend to be the optimum for exoPG, such as the exoPGs from *Aspergillus sojae* (pH 4.0) (Dogan and Tari, 2008) and *Aspergillus kawachii* (pH 3.0–4.0) (Byrne et al., 2017). The optimum conditions for polygalacturonase activity of the ExoPG-His 2 transformant were in the same range as the native cellulases derived from *T. reesei* (Voutilainen et al., 2008). Hence, these enzymes can function under similar conditions to hydrolyze pomelo peels.

3.4. Production of GalA by using the enzyme secreted from T. reesei transformants

The capacity to hydrolyze pomelo peels was compared between the crude enzyme secreted by *T. reesei* transformants and the RUT-C30 host strain. The amount of individual sugars, GalA (from polygalacturonase activity), glucose, cellobiose (generated from cellulolytic enzymes), and xylose (from xylanase), were determined by HPLC. As shown in Fig. 3, glucose (33.0–49.2 mg/g biomass), cellobiose (73.3–76.5 mg/g biomass), and xylose (18.4–28.3 mg/g biomass) were produced at similar levels by the host strain and the recombinant *T. reesei* strains. Interestingly, all enzymes produced by the *T. reesei* ExoPG and ExoPG-His strains delivered at least a 5-fold higher yield of GalA (130.0–151.1 mg/g biomass) than the host strain (24.8 mg/g biomass). The highest GalA yield was achieved by the ExoPG-His 2 crude enzyme with 151.1 mg/g biomass, which was 6.1-fold higher than that of the RUT-C30 strain. This result confirms that the overexpression of *exoPG* in *T. reesei* improved the ability of its crude enzyme to extract GalA from pomelo peels. The *T. reesei* ExoPG-His 2 strain was selected for the following study.

To confirm the concept of the synergistic action between *T. reesei* cellulases and exoPG, the ability of GalA production of crude enzyme produced by *T. reesei* ExoPG-His 2 was compared to that of the recombinant exoPG enzyme (*P*exoPG) produced by the recombinant yeast strain, *P. pastoris* BMGC 210, using the same total protein loading (2.5 mg protein/g dry substrate) in the hydrolysis reaction. *P*exoPG showed only exoPG activity, with cellulase and xylanase activities absent. After pomelo peel hydrolysis, *P*exoPG yielded GalA as the major product producing 104.3 mg/g biomass, whereas glucose or cellobiose liberated via cellulase activity was not detected. Although the specific polygalacturonase activity of the crude enzyme from *T. reesei* ExoPG-His 2 enzyme (117.7 U/mg protein) was considerably was lower than that of the *P*exoPG enzymes (5,546 U/mg protein), the GalA product released by the action of ExoPG-His 2 enzyme was up to 45% higher than that of the *P*exoPG enzyme based on the same protein loading. These results suggest that efficient GalA extraction from pomelo peels requires the

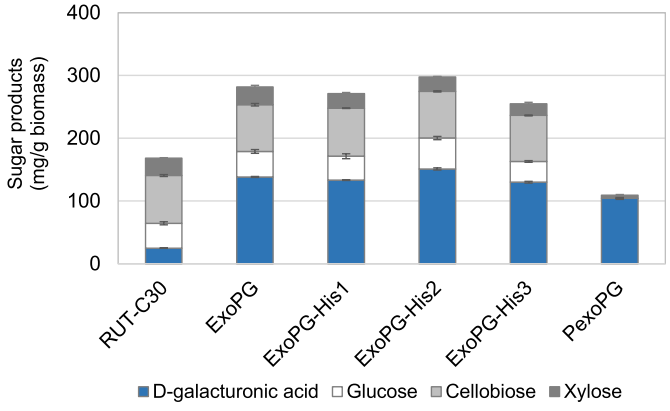

Fig. 3. Products of pomelo peel hydrolysis using enzymes from the *T. reesei* transformants (ExoPG, ExoPG-His 1, ExoPG-His 2, ExoPG-His 3), RUT-C30 host strain, and *P. pastoris* expressing exoPG (*P*exoPG).

synergy of exopolygalacturonase, cellulase, and xylanase activities.

3.5. Optimization of the production of GalA from pomelo peels

To optimize the production of GalA from pomelo peels, temperature and pH effects on the yield of GalA and other sugar products were evaluated. The maximum yields of GalA, glucose, and xylose of 142.00, 44.36, and 90.53 mg/g biomass, respectively, were obtained when the hydrolysis reaction was performed at 60 °C (Fig. 4). This result was consistent with the results obtained at the optimum temperature for exoPG-His enzyme activity. For the effect of pH, the highest GalA yield was obtained in the pH range of 5.0–6.0 (Fig. 5). Higher pH values (above 6.0) were followed by a decrease in the hydrolysis rate. The maximum GalA yield was achieved at pH 6.0, corresponding to 148.1 mg/g biomass. This study revealed that the temperature and pH of the hydrolysis reaction influence GalA yield. Subsequently, the hydrolysis of pomelo peel was performed at 60 °C and pH 6.0. After optimization, GalA yield was improved by up to 11.2%, even though the maximum GalA yield (148.1 mg/g biomass) in the present study was lower than that of a previous experiment (151.1 mg/g biomass; Fig. 3). The variation in GalA yield might be due to the pomelo peel substrates being obtained at different times. Therefore, the heterogeneity of pectin composition in plant biomass might depend on the location, fruit variety, and other environmental factors (Lara-Espinoza et al., 2018), which may affect enzymatic hydrolysis by cellulolytic and pectinolytic enzymes.

During the enzymatic hydrolysis process, the substrate (pomelo peel) concentration is an important parameter that affects the hydrolysis rate. The effect of substrate loading on the efficiency of GalA extraction was determined using 5–15% (w/v) of pomelo peel with enzyme loading at 2.5–10 mg/g dry substrate. It was found that the GalA concentration increased depending on the percentage of substrate loading (data not shown). The highest GalA yield (147.8 mg/g) was observed when the substrate loading was 5% (w/v) (Fig. 6). Increasing the substrate loading to 10% (w/v) and 15% (w/v) caused a reduction in GalA yield despite the varying enzyme loading amounts. The highest glucose yield was achieved with 10% (w/v) substrate loading. Moreover, xylose yields were not significantly different when substrate loading was 5–10% (w/v). These results indicate a strong end-product inhibition of polygalacturonase by its monomeric product (Kiss et al., 2009). The use of high substrate concentrations to yield high product concentrations is preferable in the biorefinery industry as the capital and operating costs of the hydrolysis process can be minimized in this manner (Leh et al., 2017). The application of a membrane bioreactor in pectin hydrolysis by polygalacturonase could reduce the product inhibition effect and

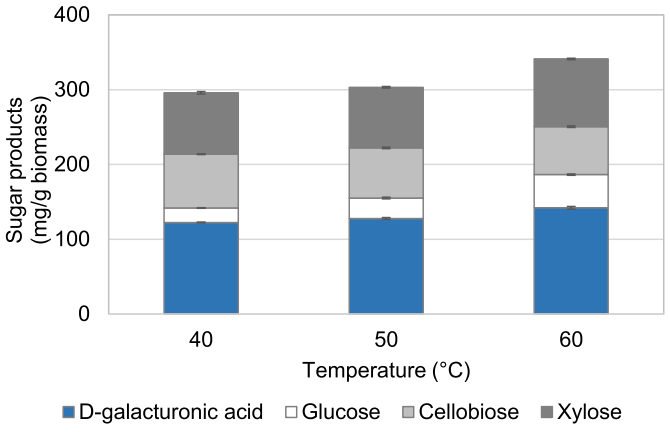

Fig. 4. Effect of temperature on the hydrolysis of pomelo peels. The reaction contained 5% (w/v) of pomelo peel, 50 mM sodium acetate buffer (pH 5.0), 1 mM sodium azide, and 2.5 mg protein/g biomass of enzyme loading. The reaction was terminated after 48 h of incubation.

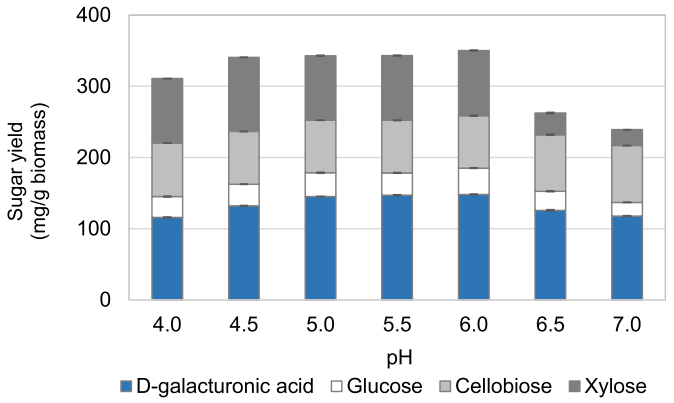

Fig. 5. Effect of pH on the hydrolysis of pomelo peels. The reaction contained 5% (w/v) of pomelo peel, 1 mM sodium azide, 2.5 mg protein/g biomass of enzyme loading. The hydrolysis experiment was conducted at 50 °C for 48 h.

improve the productivity of GalA (Bélafi-Bakó et al., 2007). Prolonged hydrolysis time might help to improve GalA production under high substrate concentration conditions (Zheng et al., 2012).

3.6. Economic assessment of the GalA production from pomelo peels

In order to evaluate the economic feasibility of the proposed process, the GalA production process from pomelo peels at commercial scale was designed and simulated based on the experimental data (Fig. S3). Two scenarios with different enzyme loading levels were simulated and evaluated for profitability. Based on the scale of 350 kg/d pomelo peel feeding, crushed pomelo peel subjected to hydrolysis process. The cost distribution and total product sales of these two scenarios were summarized in Table 2. The total capital cost and total operating cost including raw materials, utilities, and maintenance for both scenarios were similar, and calculated to approximately 0.878 M USD/y and 0.936 M USD/y, respectively. This led to the nearly identical of total investment cost obtained for both scenarios owing to the similar unit operation cost applied. The only difference for these two scenarios is the productivity of GalA. For the scenario I, the total GalA production rate was 47.97 kg/d and the annual GalA production rate was 345.36 t/y. The scenario II showed 51.74 kg/d of the total GalA production rate and 372.51 t/y of the annual production rate. These results indicate that the amount of enzyme loading affects directly to the GalA production yield. Increasing enzyme loading from 2.5 to 10 mg/g dry substrate could improve the GalA production yield up to 8%. As a consequence, the total product sales for the scenario II was higher than the scenario I. Given the economic results, the profitability of the payback period for both scenarios were evaluated. Both scenarios are economically feasible because the capital investment can be returned in less than 1 year (0.7 y for scenario I and 0.5 y for scenario II). This short payback period depends on the average cash flow for certain year and the total capital investment cost.

The GalA production strategy developed here is promising for further applications. In the present study, GalA yields of 147.8–151.1 mg/g biomass were achieved from the hydrolysis of 5% (w/v) pomelo peel by the crude enzyme from *T. reesei* expressing exoPG. In the previous study, the production yield of GalA from hydrolysis of sugar beet pulp has been reported to be approximately 97.8 mg/g biomass [8.8 g/L GalA from 9% (w/v) sugar beet pulp] using an *Aspergillus niger* crude enzyme (Schafer et al., 2020), which is lower than the yield obtained in the current study. Therefore, the difference in biomass substrates might be responsible for the varying GalA yield. Enzymatic conversion of citrus pectin into GalA by *Aspergillus oryzae*-fermented solids generated up to 247 mmol/L of GalA (Leh et al., 2017), higher than the GalA concentration obtained in this study. However, pectin powder that they used as substrate is less complex than pomelo peel or other pectin-rich biomasses, and its

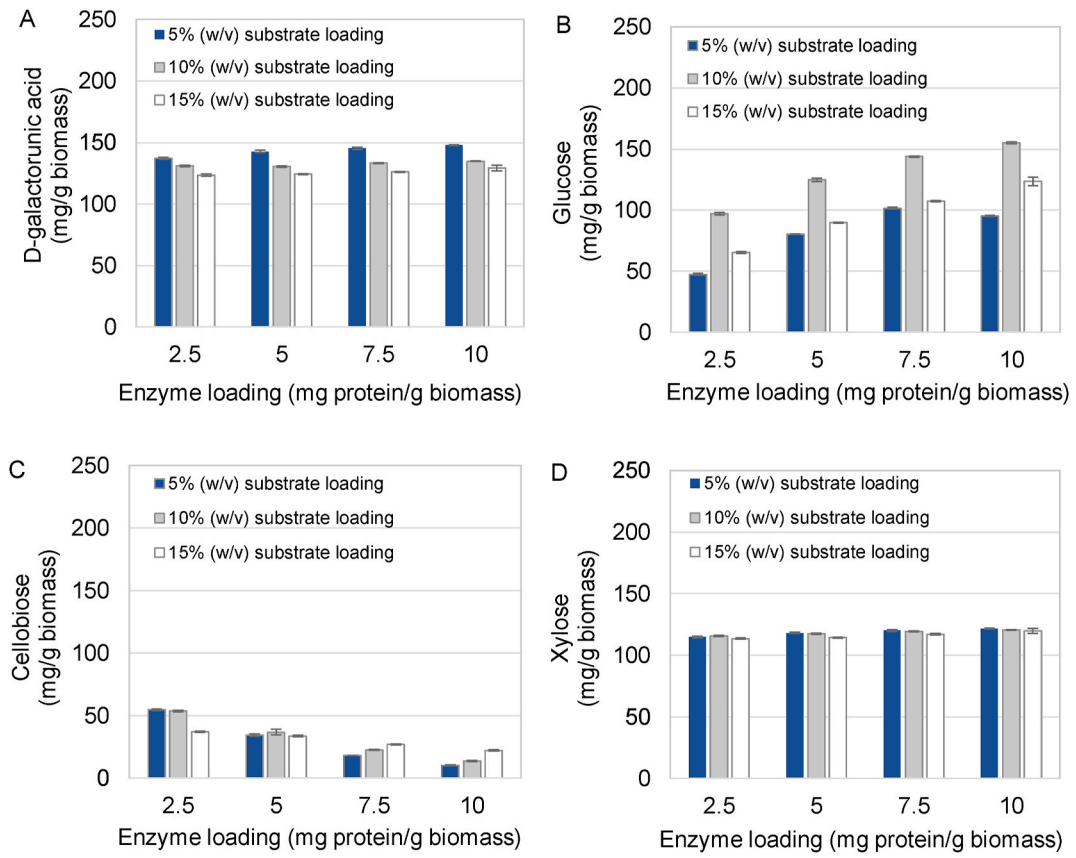

Fig. 6. Effects of the enzyme and pomelo peel concentration on the production of GalA and other sugars. The reaction was incubated at 60 °C for 48 h.

Table 2
Cost distribution and total product sales of scenario I and II of GalA production.

Summary	Scenario I Enzyme loading at 2.5 mg protein/g dry substrate	Scenario II Enzyme loading at 10 mg protein/g dry substrate
Total Operating cost (M USD/Year)	0.878	0.936
- Raw material cost	0.728	0.786
- Utilities cost	0.041	0.041
- Maintenance cost	0.109	0.109
Total Capital cost (M USD)	3.516	3.522
- Equipment cost	0.294	0.294
- Installed cost	0.088	0.088
- Build & Facility cost	2.337	2.337
- Engineering cost	0.408	0.408
-Contingency cost	0.390	0.395
Total Product Sales (M USD/Year)	17.211	18.563
Payback Period (Year)	0.7	0.5

preparation required a harsh thermochemical extraction process using nitric acid (100 °C and pH 1.5) (Vriesmann et al., 2011). In this one-step process, enzyme biocatalyst is biodegradable and the process can be performed at mild condition (60 °C and pH 6.0). As a result, the use of toxic substance like strong acid can be avoided. Therefore, the condition can be considered as a greener process. Furthermore, the direct extraction of GalA from a cheap and renewable pectin-rich biomass using the developed enzymatic process can reduce the cost of pectin preparation, promote sustainable production of GalA, and decrease environmental problem generated by disposal of the agricultural waste. From this perspective, the one-step enzymatic approach reported here will promote a much eco-friendly and economical production of GalA. Moreover, the recombinant *T. reesei* expressing exoPG constructed in the present study could also be applied to the fruit juice extraction process as well as the production of animal feeds, which require a combination of pectinase and cellulase activities (Jayani et al., 2005).

Further genetic manipulation of the *T. reesei* strain to overexpress other pectinases, such as endopolygalacturonase or pectin methylesterase, should be conducted to improve GalA yield from pectin-rich material. Endopolygalacturonase (EC 3.2.1.15) hydrolyzes the inner linkages within the homogalacturonan molecules of pectin (Latarullo et al., 2016), resulting in the release of the free end of the homogalacturonan polymer, which is digested by exoPG into the final GalA product. Pectin methylesterase (EC 3.1.1.11) is responsible for removing methyl groups from pectin to provide access to depolymerizing enzymes, such as exoPG, thereby playing an important role in determining the extent of degradation of the pectin polysaccharides (Ünal and Şener, 2015). Recently available *T. reesei* genetic engineering toolboxes including site-directed gene insertion (Derntl et al., 2015) and genome editing system (Liu et al., 2015) allow for developing this fungal strain.

To the best of our knowledge, this is the first demonstration of the use of recombinant *T. reesei* overexpressing exoPG to produce GalA from pomelo peel. The complexity of plant cell walls is due to the association of cellulose, xyloglucan, and pectin (Van de Wouwer et al., 2018). Therefore, to obtain high GalA yields from pomelo peel, an enzyme cocktail comprising cellulase, xylanase, and pectinase is necessary to destroy plant cell walls and pectin hydrolysis. This study clearly demonstrated that the combined activity of exoPG and cellulases could enhance the yield of GalA extraction by approximately 45% compared with the activity of exoPG (*P*exoPG) alone. The sugar products released from pomelo peel hydrolysis, including glucose, cellobiose, and xylose, can be used as substrates for the production of bioethanol, xylitol, or other high value-added products by fermenting yeast strains, such as *Saccharomyces cerevisiae* (Casa-Villegas et al., 2018) and *Kluyveromyces marxianus* (Feng et al., 2021). In this way, the recombinant fungal strain

developed in this study would be useful for not only production of GalA, but also production of a large variety of bio-based chemicals for sustainable development.

From the environmental aspects, the enzymatic process developed in this study could be considered as a clean production process as the reaction was undertaken under mild conditions. Biocatalysis processes by using enzymes are in the context of green chemistry as enzymes is a green, natural, and renewable catalyst (Shoda et al., 2016). Even though environmental impact of this process cannot be determined at this stage, it should be point out that the enzymatic process developed in this study is more environmentally friendly comparing with the traditional chemical method. However, more detailed evaluation on environmental impact will be performed in future studies.

4. Conclusion

T. reesei RUT-C30, an industrial strain for cellulase production, was used as the host strain for expressing exoPG from *A. aculeatus* to obtain an enzyme mixture of cellulase and exoPG for producing GalA from pectin-rich agricultural wastes. The enzyme secreted by the recombinant strain *T. reesei* ExoPG-His 2 exhibited 64.71 U/mL of polygalacturonase activity, which was approximately 4-fold higher than that of the host strain, while the background cellulase activity of *T. reesei* ExoPG-His 2 was comparable to that of the original strain. For GalA production from pomelo peel, *T. reesei* overexpressing exoPG-His enzyme delivered an up to 6.1-fold higher yield than that of the host strain's enzyme. The economic analysis revealed that the developed GalA production process was profitable. Therefore, this study provides an eco-friendly strategy for clean production of GalA and pectin-rich agricultural waste biorefinery to aid in achieving a sustainable bioeconomy. Further optimization of the fungal strain development and fermentation process will lead to higher GalA yield for its sustainable production using enzymatic method.

CRediT authorship contribution statement

Chatuphon Siamphan: Investigation, Writing. **Jantima Arnthong:** Investigation, Writing. **Sudarat Tharad:** Writing, Visualization. **Fei Zhang:** Methodology. **Jie Yang:** Writing. **Thanaporn Laothanachareon:** Investigation. **Santi Chuetor:** Formal analysis, Writing. **Verawat Champreda:** Funding acquisition, Writing – review & editing. **Xin-Qing Zhao:** Conceptualization, Supervision, Writing – review & editing. **Surisa Suwannarangsee:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations in this study

exoPG	exopolygalacturonase
GalA	D-galacturonic acid
PDC1	pyruvate decarboxylase 1
CBH1	cellobiohydrolase 1
His	histidine
LB	Luria-Bertani
PCR	polymerase chain reaction
MM	Mandels medium
BSA	bovine serum albumin
YPD	yeast peptone dextrose
YNB	yeast nitrogen base
BMMY	buffered complex methanol medium
FPase	filter paper activity
CMCase	carboxymethyl cellulase
DNS	3,5-dinitrosalicylic acid
BGL	β-glucosidase
pNPG	p-nitrophenyl-β-D-glucopyranoside
HPLC	high-performance liquid chromatograph
*P*exoPG	Recombinant exoPG enzyme produced by *P. pastoris*

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2021.129958.

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